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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Activated PAR-2 Regulates Pancreatic Cancer Progression through ILK/HIF-α—Induced TGF-α Expression and MEK/VEGF-A—Mediated Angiogenesis

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Tissue factor initiates the process of thrombosis and activates cell signaling through protease-activated receptor-2 (PAR-2). The aim of this study was to investigate the pathological role of PAR-2 signaling in pancreatic cancer. We first demonstrated that activated PAR-2 up-regulated the protein expression of both hypoxia-inducible factor-1α (HIF-1α) and HIF-2α, resulting in enhanced transcription of transforming growth factor-α (TGF-α). Down-regulation of HIFs-α by siRNA or YC-1, an HIF inhibitor, resulted in depleted levels of TGF-α protein. Furthermore, PAR-2, through integrin-linked kinase (ILK) signaling, including the *p*-AKT, promoted HIF protein expression. Diminishing ILK by siRNA decreased the levels of PAR-2—induced *p*-AKT, HIFs-α, and TGF-α; our results suggest that ILK is involved in the PAR-2—mediated TGF-α via an HIF-α—dependent pathway. Furthermore, the culture medium from PAR-2—treated pancreatic cancer cells enhanced human umbilical vein endothelial cell proliferation and tube formation, which was blocked by the MEK inhibitor, PD98059. We also found that activated PAR-2 enhanced tumor angiogenesis through the release of vascular endothelial growth factor-A (VEGF-A) from cancer cells, independent of the ILK/HIFs-α pathways. Consistent with microarray analysis, activated PAR-2 induced *TGF-A* and *VEGF-A* gene expression. In conclusion, the activation of PAR-2 signaling induced human pancreatic cancer progression through the induction of TGF-α expression by ILK/HIFs-α, as well as through MEK/VEGF-A—mediated angiogenesis, and it plays a role in the interaction between cancer progression and cancer-related thrombosis. *(Am J Pathol 2013, 183: 566—575; http://dx.doi.org/10.1016/j.ajpath.2013.04.022)*

The association between thrombosis and malignancies is known as Trousseau syndrome, and advanced pancreatic cancer has been associated with venous thromboembolism in epidemiological studies. The up-regulation of tissue factor (TF), the primary initiator of coagulation, is considered a biomarker for the development of thrombosis and cancer progression. TF overexpression can result from the mutation of oncogenes, such as *KRAS* and *p53*, and it is associated with tumor progression and angiogenesis in colorectal cancer. The expression of TF has also been related to staging, overall survival, microvessel density, metastasis, and the rate of thrombosis of patients with cancer. TF functions by binding to factor VII (FVII), leading to the activation of the coagulation cascade and protease-activated receptor (PAR)-2 signaling and integrin ligation, which influence cancer progression, such as cell survival, angiogenesis, and invasion.[1]

The PARs are members of the GPCR family, and they are activated by different proteases, including thrombin, trypsin, and coagulant factors. PAR-1, PAR-3, and PAR-4 are thrombin-activated receptors, whereas PAR-2 is activated by trypsin. This diversity allows PARs to act in a broad spectrum of pathological capacities in different diseases, including cancers,[2] such as colorectal[3] and pancreatic[4] cancers. The

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activation of PAR-2 has been shown to increase tumor cell proliferation and invasion via mitogen-activated protein kinase (MAPK) activation, and to stimulate angiogenesis by releasing vascular endothelial growth factor-A (VEGF-A).[5,6] Furthermore, under hypoxic conditions, tumor cells release microvesicles containing TF, which activates PAR-2 signaling in endothelial cells (ECs) and subsequently induces angiogenesis.[7] These findings demonstrate that TF/FVII/PAR-2 signaling promotes tumor angiogenesis through the release of VEGF from tumors and the activation of ECs by PAR-2.

Integrin-linked kinase (ILK) is an integrin-interacting protein that possesses both serine/threonine protein kinase activity and adaptor properties. The interaction of ILK with the cytoplasmic tail of β1 integrin, which is ligated with TF and PAR-2, induces survival signaling through the phosphorylation of AKT (Ser-473) and glycogen synthetase kinase (GSK)-3β (Ser-9).[8] Several studies have indicated that ILK is overexpressed in various types of cancers and is correlated with a poor patient prognosis.[9] ILK also increases hypoxia-inducible factor (HIF)-1α accumulation by activating the AKT/mammalian target of rapamycin pathway and inducing transcription of VEGF-A, which plays a role in regulating angiogenesis.[10] Moreover, a recent study showed that ILK interacts with centrosomal proteins and causes mitotic spindle reorganization.[11] As a result, ILK regulates cancer progression through its effects on integrin signaling and its actions as a mitotic regulator.

The HIFs are hallmarks of the hypoxic tumor microenvironment. There are two subunits of HIFs: O_2-sensing α-subunits (HIF-1α, HIF-2α, and HIF-3α) and a stable β-subunit (HIF-1β). In many human cancers, both HIF-1α and HIF-2α are correlated with angiogenesis and poor patient prognosis, but they function through different mechanisms to regulate tumor growth. However, the role of HIF-3α remains unclear. Several studies have shown that HIF-1α, but not HIF-2α, enhances the transcription of glycolytic pathway genes to raise energy uptake. In contrast, HIF-2α, which is highly expressed in tumor-initiating and stem cell–like cells, acts to directly increase the activity of octamer-binding transcription factor 4, which maintains stem cells in an undifferentiated state. The switch in the expression of HIFs may affect cancer progression and angiogenesis because of the involvement of HIF-1α and HIF-2α in different stages of tumor progression and vascular development. Therefore, targeting of HIFs is an important potential therapeutic method in solid tumors.[12–14]

The tumor microenvironment is capable of inducing growth factor expression and survival signals through the AKT/mammalian target of rapamycin and Ras/Raf/MEK/extracellular signal–regulated kinase (ERK) pathways. This results in cancer cell growth and the expression of angiogenic factors that control the tumor angiogenic switch, including VEGF-A, fibroblast growth factor, platelet-derived growth factor, and insulin-like growth factor. Increased tumor-associated angiogenesis also results in improved tumor growth and metastasis. Therefore, the disruption of communication between cancer cells and the tumor microenvironment is a viable therapeutic strategy to target angiogenesis.[15]

PAR-2 has previously been reported to be associated with cell proliferation and cyclooxygenase-2 expression in pancreatic cancer cells.[4] In one of our previous studies, we found that PAR-1 activation had an effect on the accumulation of HIF-1α and twist protein, which acts to regulate cell motility.[16] Therefore, we interestingly investigated if PAR-2 activation may have some pathological effects on pancreatic cancer. We report that the activation of PAR-2 causes an increase in HIF-α–regulated transforming growth factor (TGF)-α expression via ILK signaling, as well as enhanced angiogenesis via MEK-ERK–mediated VEGF-A expression.

Materials and Methods

Cell Culture

Human pancreatic cancer cell lines AsPC-1 and BxPC-3, human colorectal cancer cell line HCT-116, and human umbilical vein endothelial cells (HUVECs) were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). Human pancreatic and colorectal cancer cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (v/v). HUVECs were maintained in M199 contained with 20% fetal bovine serum (v/v). Both media were supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Cells were maintained in a humidified incubator at 37°C in 5% CO_2/95% air.

Reagents

RPMI 1640 medium, M199 medium, fetal bovine serum, penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). LY294002, PD98059, SB203580, SP600125, and nucleolin antibody were ordered from Sigma Chemical (St. Louis, MO). $CoCl_2$ was purchased from Wako (Osaka, Japan). PAR-2–activating peptide (PAR-2 AP), SLIGKV-NH_2, was ordered from Bachem (Bubendorf, Switzerland). TRIzol reagent was from Invitrogen (Carlsbad, CA), random primer and M-MLV RT were purchased from Promega (Madison, WI), and pro-Teq was from Protech (Taipei, Taiwan). Antibodies against HIF-1α and Matrigel basement membrane matrix were purchased from BD Biosciences (San Jose, CA). Phosphorylated (Ser-473) AKT was bought from Epitomics (Burlingame, CA). Antibodies specific for total AKT, phosphorylated (Ser-9) or total GSK-3β, phosphorylated (Thr-202/Tyr-204) or total p44/p42 MAPK, ILK, pro-TGF-α, and HIF-2α antibodies were obtained from Cell Signaling Technology (Beverly, MA). Actin antibody was purchased from Chemicon (Billerica, MA). Antibodies to horseradish peroxidase–conjugated anti-mouse and anti-rabbit IgGs were ordered from Santa Cruz Biotechnology (Santa Cruz, CA).

Microarray Analysis

BxPC-3 cells were treated with or without PAR-2 AP for 4 hours. Total RNAs were harvested by TRIzol reagent, followed by purification using an RNeasy Mini kit (Qiagen, Valencia, CA). Samples were processed and performed according to the procedure of Human OneArray from Phalanx Biotech (Hsinchu). The results were analyzed by the Rosetta Resolver System (Rosetta Biosoftware, Seattle, WA). Compared with the control sample, the fold change of gene expression is duplicated and established at $\log_2 |\text{fold change}| \geq 0.5$ and $P < 0.05$, by using DAVID Bioinformatic Resources (NIH, Bethesda, MD) subjected to pathway and gene ontology analysis. Microarray data is has been uploaded to the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE44827).

RT-PCR Data

Total RNA was isolated with TRIzol reagent by a standard protocol. mRNA (5 μg) was incubated with random primer at 65°C for 5 minutes, and then reacted with M-MLV RT at 37°C for 1 hour to obtain cDNA. The primer sets were as follows: TF, 5′-CTACTGTTTCAGTGTTCAAGCAGTGA-3′ and 5′-CAGTGCAATATAGCATTTGCAGTAGC-3′; PAR-2, 5′-TGCTGGGGGCCGCCATCCTGCTA-3′ and 5′-ATAGGCAATCTTCAAGGGGAACC-3′; VEGF-A, 5′-GAAGTGGTGAAGTTCATGGATGTC-3′ and 5′-CGATCGTTCTGTATCAGTCTTTCC-3′; TGF-α, 5′-TCGCTCTGGGTATTGTGTTG-5′ and 5′-GACCTGGCAGCAGTGTATCA-3′; and actin, 5′-GGTGGCTTTTAGGATGGCAAG-3′ and 5′-ACTGGAACGGTGAAGGTGACAG-3′. For TF and VEGF-A, a PCR was performed with 30 cycles, with denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 45 seconds. For TGF-α and actin, 30 cycles of amplification were done under the following conditions: 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 40 seconds. For PAR-2, the reaction condition was performed with 30 cycles at 95°C, 54°C, and 72°C for 30 minutes. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide.

Western Blot Analysis

Whole cell lysate was extracted by radioimmunoprecipitation assay buffer containing Tris (50 mmol/L), sodium chloride (150 mmol/L), SDS (0.1%), sodium deoxycholate (0.5%), and NP-40 (1%). Nuclear extraction and Western blot analysis were used as described previously.[16]

Transient Transfection

We used validated siRNA directed against HIF-1α, HIF-2α, and ILK mRNA from Ambion (Carlsbad). BxPC-3 cells were trypsinized and 10^6 cells were counted into 100 μL of Nucleofector Solution (Amaxa/Lonza, Allendale, NJ) with 100 nmol/L siRNA. Transfection was performed using Nucleofector II (Amaxa/Lonza), according to the manufacturer's instructions. The transfected cells were seeded into 6-well plates for 48 hours and then prepared for further analysis.

VEGF-A ELISA

AsPC-1, BxPC-3, and HCT-116 cells were seeded as 150,000 cells per well into a 24-well plate for overnight analysis. Cells were treated with the indicated concentration of FVIIa or PAR-2 AP for 24 hours, and then the culture medium was collected. The amount of VEGF-A in culture medium was detected by a Human VEGF-A Quantikine ELISA (enzyme-linked immunosorbent assay) Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

HUVEC Proliferation Assay

For preparation of conditioned medium, BxPC-3 cells were treated with or without PAR-2 AP for 24 hours, and then conditioned medium was collected. HUVECs were seeded as 5000 cells per well into a 96-well plate for overnight analysis. After cells adhered, culture medium was replaced by conditioned medium for 48 hours. HUVEC proliferation was measured by 0.5% crystal violet with methanol (4:1). Crystal violet was solubilized with 0.1 mol/L citric acid, and absorbance was detected at a wavelength of 550 nm.

Tube Formation

A 96-well plate was coated with 60 μL Matrigel (BD Bioscience) and incubated in 37°C for 1 hour before starting to subculture HUVECs. After subculturing and counting 20,000 HUVECs, cells were resuspended into conditioned medium and seeded onto a Matrigel-coated well for 24 hours. The mean of tube length was measured by ImageJ version 1.46r software (NIH) in five areas randomly using a microscope.

Statistics

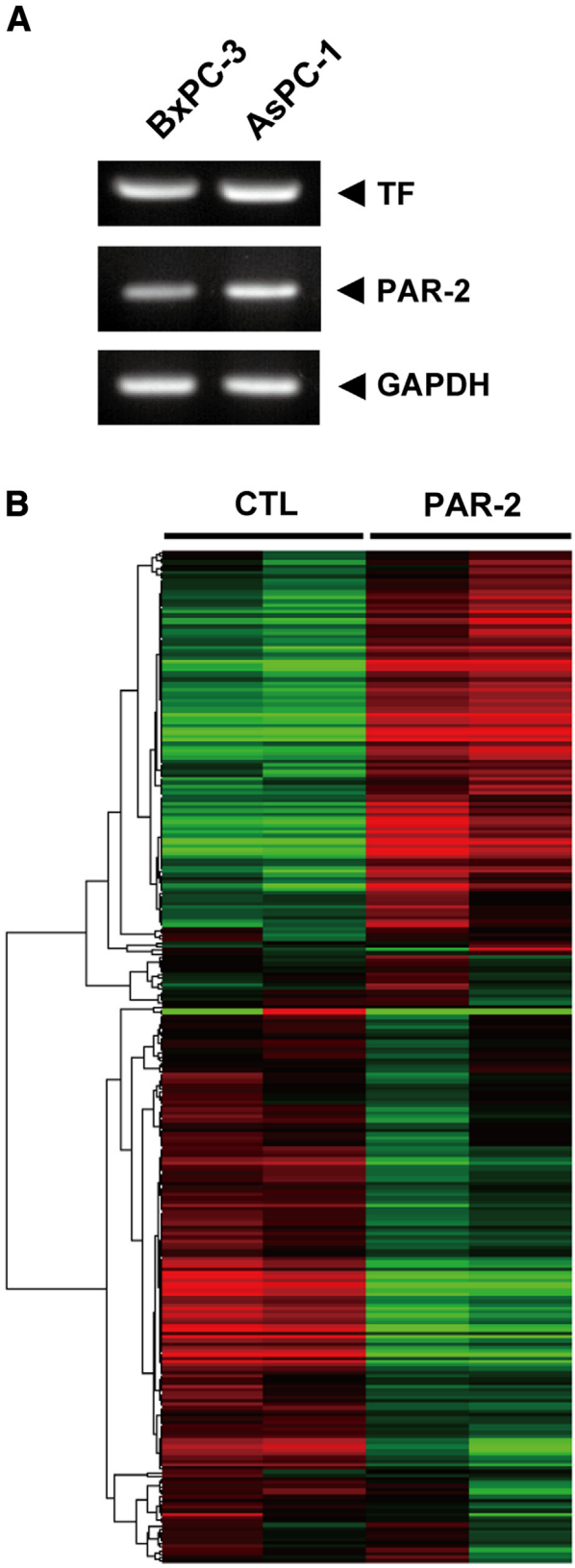
Every experiment was performed at least three times. All data represent means ± SEM from three independent experiments. Statistical analysis was performed using a Student's *t*-test.

Results

PAR-2 AP Induces Gene Expression in TF/PAR-2–Expressing BxPC-3 Cells

Many studies have demonstrated that the activation of TF/PAR-2 signaling regulates tumor proliferation, angiogenesis, and metastasis.[1] In this study, we investigated the expression of TF and PAR-2 in pancreatic cancer cell lines BxPC-3 and AsPC-1, and we found that TF and PAR-2 mRNAs were constitutively expressed in both cell lines (Figure 1A). Gene profiling may be used as an approach to investigate the different physiological and pathological roles of PAR-2

signaling, and microarray-based gene expression studies have shown that PAR-2 regulates several genes in human breast cancer cells and human kidney cells, including cytokines, chemokines, and growth factors.[17,18] Therefore, we examined the profile of genes expressed in response to PAR-2 AP in pancreatic cancer BxPC-3 cells by microarray analysis; a cluster analysis of PAR-2 AP-regulated genes is shown in Figure 1B. By using DAVID Bioinformatic Resources, we found that PAR-2 AP-mediated genes can be overlaid on the renal cell carcinoma pathway (Kyoto Encyclopedia of Genes and Genomes hsa04960), which involves the deregulation of multiple oncogenes. In particular, HIF, which is a major transcription factor in renal cell carcinoma, influences cancer progression owing to the absence of Von Hippel—Lindau disease.[19] The microarray results showed that PAR-2 induced the up-regulation of HIF-α—activated target genes, such as glucose transporter 1 (GLUT1; *SLC2A1*), VEGF-A, and TGF-α; however, HIF-2α (*EPAS1*) was down-regulated (Table 1). The activation of MAPK signaling by PAR-2 subsequently targets the AP-1 and Ets-1 transcription factor families. As shown in Table 1, the expression of MAPK1, Ets-1, and c-Jun was increased in BxPC-3 cells treated with PAR-2 AP, suggesting that the activation of PAR-2 could regulate HIF-α— and MAPK-mediated signaling in human pancreatic cancer cells.



Activation of PAR-2 Increases the Expression of the α Forms of HIFs, Pro—TGF-α, and VEGF-A in Human Pancreatic Cancer Cells

Previous studies have shown that the activation of PAR-1 increases HIF-1α protein levels in human colorectal cancer cells.[16] The results of this study suggest that the HIF pathway is involved in PAR-2—activated human pancreatic cancer cell functions. Next, we proved that PAR-2 AP significantly induced HIF-1α and HIF-2α protein expression in a concentration-dependent manner in BxPC-3 and AsPC-1 human pancreatic cancer cell lines (Figure 2, A and B). In addition, HIF-1α protein expression was also up-regulated by PAR-2 AP in human colorectal cancer HCT-116 cells (Figure 2A). $CoCl_2$ was used as a positive control. Furthermore, FVIIa-induced TF/FVII/PAR-2 signaling increased HIF-1α and HIF-2α protein expression in both pancreatic cancer cell lines (Figure 2C). These results indicate that activation of PAR-2 can induce HIF-α protein accumulation in human cancer cells under normoxic conditions.

To validate the results of our microarray analysis, we next performed RT-PCR assays to confirm the changes in TGF-α and VEGF-A mRNA expression. The results showed that PAR-2 AP significantly induced TGF-α and VEGF-A mRNA

Figure 1 Hierarchical clustering of gene profiles in TF- and PAR-2—expressing pancreatic cancer cell lines. **A:** RT-PCR analysis of TF- and PAR-2 mRNA levels. BxPC-3 and AsPC-1 were grown in the culture medium. **B:** Clustering analysis of microarray data comparing BxPC-3 cells with those treated with PAR-2 AP. Total RNA of BxPC-3 cells treated with or without 100 μmol/L PAR-2 AP for 4 hours was extracted and analyzed by Human OneArray. Genes significantly different, with $P < 0.05$ after the treatment, were pooled and used to generate heat maps. Up-regulated and down-regulated genes are represented in red and green, respectively. CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Renal Cell Carcinoma Pathway Genes (Kyoto Encyclopedia of Genes and Genomes hsa04960) Are Regulated by PAR-2 AP in BxPC-3 Cells

Accession no.	Gene symbol	Gene name	Log_2 (ratio)	*P* value
NM_001430.4	*EPAS1*	Endothelial PAS domain protein 1	−0.695304	0.000378
NM_005238.3*	*ETS1*	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	0.626951	0.014179
NM_002228.3	*JUN*	Jun proto-oncogene	0.607265	0.00028
NM_002755.3	*MAP2K1*	Mitogen-activated protein kinase kinase 1	0.590325	0.004437
NM_003629.3†	*PIK3R3*	Phosphoinositide-3-kinase, regulatory subunit 3 (γ)	−0.659642	0.005429
NM_002834.3	*PTPN11*	Protein tyrosine phosphatase, nonreceptor type 11	0.624667	0.000243
NM_006516.2	*SLC2A1*	Solute carrier family 2 (facilitated glucose transporter), member 1	0.54684	0.027656
NM_003236.2‡	*TGFA*	Transforming growth factor α	0.510164	0.044585
NM_001171629.1§	*VEGFA*	Vascular endothelial growth factor A	0.514638	0.003685

BxPC-3 cells were treated with 100 μmol/L PAR-2 AP for 4 hours before total RNA was harvested for microarray analysis. Each unique splice variant of the gene was also tested. Different accession numbers are given.

*ETS1: NM_005238.3, NM_001162422.1, and NM_001143820.1.

†PIK3R3: NM_003629.3 and NM_001114172.1.

‡TGFA: NM_003236.2 and NM_001099691.1.

§VEGFA: NM_001171629.1, NM_001171626.1, NM_001171625.1, NM_001171624.1, NM_001171623.1, NM_001033756.2, NM_001025368.2, NM_001025367.2, NM_003376.5, NM_001025366.2, NM_001171627.1, and NM_001025369.2.

PAS, PER-ARNT-SIM.

expression (Figure 2D), as well as pro−TGF-α protein expression in human pancreatic cancer cells (Figure 2E). In addition, PAR-2 AP caused AsPC-1, BxPC-3, and HCT-116 cells to release VEGF-A into the culture medium in a concentration-dependent manner (Figure 2F). Moreover, activation of TF/FVII/PAR-2 induced similar effects in pancreatic cancer cell lines, including increase of pro−TGF-α (Figure 2E) and VEGF-A (Figure 2G). Taken together, these results suggest that PAR-2 signaling significantly induces the expression of HIF-α proteins, pro−TGF-α protein, and VEGF-A in human pancreatic cancer cells.

PAR-2 AP Induces HIF-α−Mediated Production of TGF-α But Not VEGF-A

Both TGF-α and VEGF-A are known to be target genes of the transcription factors HIF-1α and HIF-2α.[12] However, in this study, a reduction in the expression levels of the HIF-α proteins (HIF-1α and/or HIF-2α) in response to siRNA treatment (Figure 3A) correlated with a decrease in pro−TGF-α protein expression (Figure 3B), but not VEGF-A production (Figure 3C), after PAR-2 AP treatment. Interestingly, a reduction in HIF-1α levels also resulted in increased nuclear expression of HIF-2α, although siRNA targeting of HIF-2α did not have an effect on the nuclear localization of the HIF-1α protein (Figure 3A). This finding indicates an unknown relationship between HIF-1α and HIF-2α. In addition, we used a well-known HIF inhibitor, YC-1, to investigate the relationship between HIFs and TGF-α. As shown in Figure 3D, YC-1 reduced PAR-2 AP-induced HIF-1α and HIF-2α protein expression in a concentration-dependent manner, which directly correlated with the observed TGF-α expression pattern in human pancreatic BxPC3 cancer cells. Taken together, these data illustrate that PAR-2 AP-mediated TGF-α production occurs via the HIFs (HIF-1α and HIF-2α) signaling pathway.

PAR-2 Activation Regulates TGF-α Production through ILK/HIF-α Signaling

Previous research has demonstrated that the HIF-1α−VEGF-A pathway is regulated by ILK in prostate cancer.[10] We first determined that ILK protein is expressed in both BxPC-3 and AsPC-1 pancreatic cancer cell lines (Figure 4A). Once activated by integrin or growth factors, ILK is capable of directly phosphorylating its downstream substrates, including *p*-AKT (Ser-473) and *p*-GSK-3β (Ser-9). We observed that PAR-2 AP up-regulated the protein expression of *p*-AKT, *p*-GSK-3β, and *p*-ERK (Figure 4B). Although *p*-ERK is not a substrate of ILK, it functions in a signaling pathway downstream of activated PAR-2. Then, the down-regulation of ILK by ILK-specific siRNA partially inhibited PAR-2 AP-mediated *p*-AKT expression, although it did not effectively inhibit PAR-2 AP-induced *p*-GSK-3β or *p*-ERK protein expression (Figure 4C). We, therefore, suggest that PAR-2 AP-activated ILK signaling functions via *p*-AKT to influence cellular phenotypes.

We further determined whether PAR-2 induced HIF-mediated TGF-α production or the release of VEGF-A via an ILK-dependent pathway. Our results demonstrated that an siRNA-mediated decrease in ILK expression led to reduced levels of both HIF-α and TGF-α proteins (Figure 4D), but it did not attenuate the release of VEGF-A in BxPC-3 cells (Figure 4E). Thus, we proved that ILK is involved in the PAR-2−mediated production of TGF-α via the HIF-α−dependent pathway in human pancreatic cancer cells.

PAR-2 AP-Treated Conditioned Medium Enhances HUVEC Proliferation and Tube Formation via an MEK-Dependent Pathway

Our results showed that PAR-2−induced VEGF-A protein was not regulated by ILK and HIF-α; however, previous

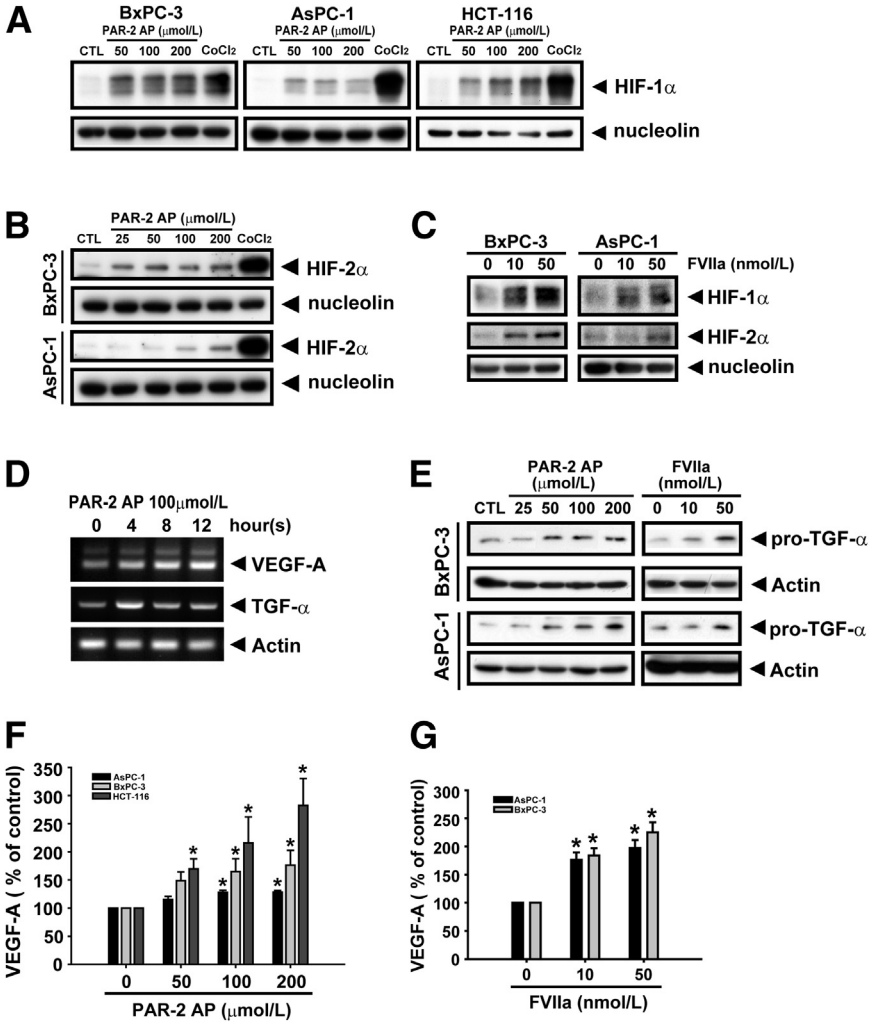


Figure 2 PAR-2 AP increases the expression of HIFs-α and their downstream target genes. Nuclear extracts were subjected to immunoblot with HIF-1α— and HIF-2α—specific antibodies. The level of nucleolin protein in the lysates was used as a loading control (CTL). **A:** BxPC-3, AsPC-1, and HCT-116 cells were treated with PAR-2 AP at the indicated concentrations or with 100 μmol/L $CoCl_2$ as a positive control for 4 hours. **B:** HIF-2α, BxPC-3, and AsPC-1 were detected using cell lines treated with PAR-2 AP at the indicated concentrations or with 100 μmol/L $CoCl_2$, as a positive control, for 4 hours. **C:** BxPC-3 and AsPC-1 cell lines were treated with FVIIa at the indicated concentrations for 4 hours. **D:** BxPC-3 cells were treated with 100 μmol/L PAR-2 AP for the indicated time interval before VEGF-A and TGF-α mRNA levels were determined by RT-PCR analysis. **E:** BxPC-3 and AsPC-1 cells were treated with PAR-2 AP or FVIIa at the indicated concentration for 24 hours, and whole cell lysates were subjected to immunoblot with antibodies against pro—TGF-α and actin. **F:** AsPC-1, BxPC-3, and HCT-116 cells were treated with PAR-2 AP at the indicated concentration. **G:** AsPC-1 and BxPC-3 cells were treated with FVIIa at the indicated concentration. After 24 hours, VEGF-A levels in the media were determined using an ELISA kit. $^*P < 0.05$.

studies have shown that PAR-2 regulates VEGF-A expression through the MAPK pathway in human breast cancer cells[5] and human glioblastoma cells.[6] We, therefore, examined whether the MAPK pathway is involved in PAR-2 AP-regulated VEGF-A release in human pancreatic cancer cells, and we found that the increase in VEGF-A release in response to PAR-2 AP is inhibited by the MEK inhibitor, PD98059 (Figure 5A). Because this finding is consistent with results from our microarray analysis, we suggest that PAR-2 AP triggers the MEK-Ets1 pathway and, thus, enhances VEGF-A transcription. We also demonstrated that, in BxPC-3 and HCT-116 cells, PAR-2 AP increases the release of VEGF-A into the culture medium (Figure 2E).

Next, we investigated whether the PAR-2—mediated release of VEGF into a tumor-conditioned medium stimulates ECs to proliferate and differentiate in an *in vitro* angiogenesis model. As shown in Figure 5, B and C, PAR-2 AP-treated tumor-conditioned medium significantly induced HUVEC proliferation and enhanced tube formation, both of which were attenuated by the conditioned medium containing PD98059, an MEK inhibitor. Consequently, these results demonstrate that the activation of PAR-2 promotes angiogenesis through MEK-mediated VEGF-A release.

Discussion

Cancer-related thrombosis is a well-known phenomenon in patients with pancreatic cancer. TF is a key factor for clotting, inflammation, tumor progression, and angiogenesis; the formation of a TF/VIIa complex results in the cleavage of PAR-2, a GPCR, which then modulates cellular functions, such as cell proliferation and survival. In human pancreatic cancer cells, we found that activated PAR-2 induces ILK/HIF-α signaling, thus affecting TGF-α protein expression and VEGF-A release, which ultimately leads to the induction of angiogenesis via an MEK-dependent pathway.

As previously mentioned, patients with pancreatic cancer are at a high risk of developing venous thromboembolism. Furthermore, previous research has indicated that the expression of TF, including full-length TF and alternative-splicing TF, is correlated with tumor progression, angiogenesis, and prognosis.[20–22] Herein, we examined the role

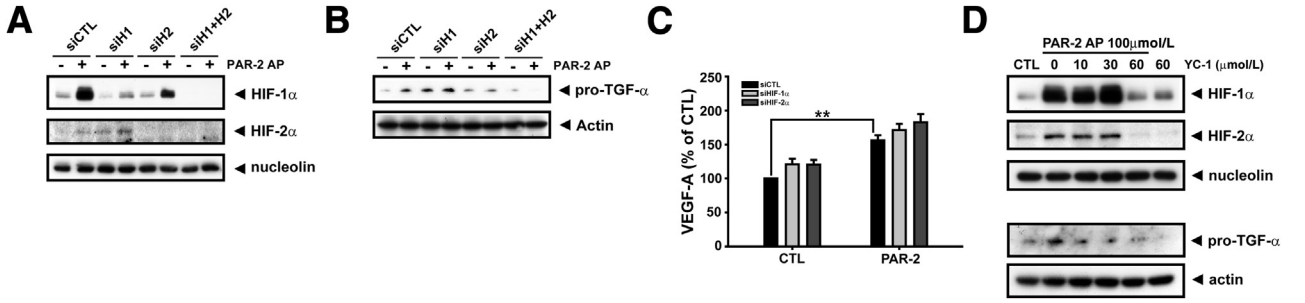


Figure 3 Depletion of HIFs-α decreases PAR-2—induced HIF-α and TGF-α expression, but it does not influence VEGF release. BxPC-3 cells were transfected with 200 nmol/L control siRNA (siCTL), siHIF-1α (siH1), or siHIF-2α (siH2) by using the Nucleofector device. **A**: BxPC-3 cells were treated with or without 100 μmol/L PAR-2 AP for 4 hours. Nuclear extracts were subjected to immunoblot analysis using specific antibodies for HIF-1α, HIF-2α, and nucleolin. **B**: BxPC-3 cells were treated with or without PAR-2 AP for 24 hours. Whole cell lysates were subjected to immunoblot with pro—TGF-α and actin primary antibodies. **C**: BxPC-3 cells were treated with or without PAR-2 AP for 24 hours before the culture media were collected and VEGF-A levels were detected by an ELISA kit. **D**: BxPC-3 cells were pretreated with YC-1 at the indicated concentrations for 30 minutes before treatment with or without 100 μmol/L PAR-2 AP at the indicated concentrations for 4 hours (**top panel**) or 24 hours (**bottom panel**). $^{**}P < 0.01$.

of PAR-2 in human pancreatic cancer by using PAR-2—AP-SLIGKV-NH_2, which can activate PAR-2 and trigger signaling pathways by mimicking the action of trypsin. Previous research has demonstrated that activated PAR-2 can influence many genes, including cell cycle regulator and inflammatory mediators in human breast cancer MDA-MB-231 cells[17] and human HEK293 kidney cells.[18] In the present study, microarray analysis revealed that PAR-2 AP regulates genes encoding proteins involved in many biological functions, including the regulation of cell growth and differentiation (MAPK family, such as JUN and MAP2K1) and tumor progression (TGF-α and VEGF-A). Furthermore, the resulting gene profile could be linked with a renal carcinoma pathway (Kyoto Encyclopedia of Genes and Genomes hsa04960) (Table 1) that involves multiple genes associated with the HIF pathway.

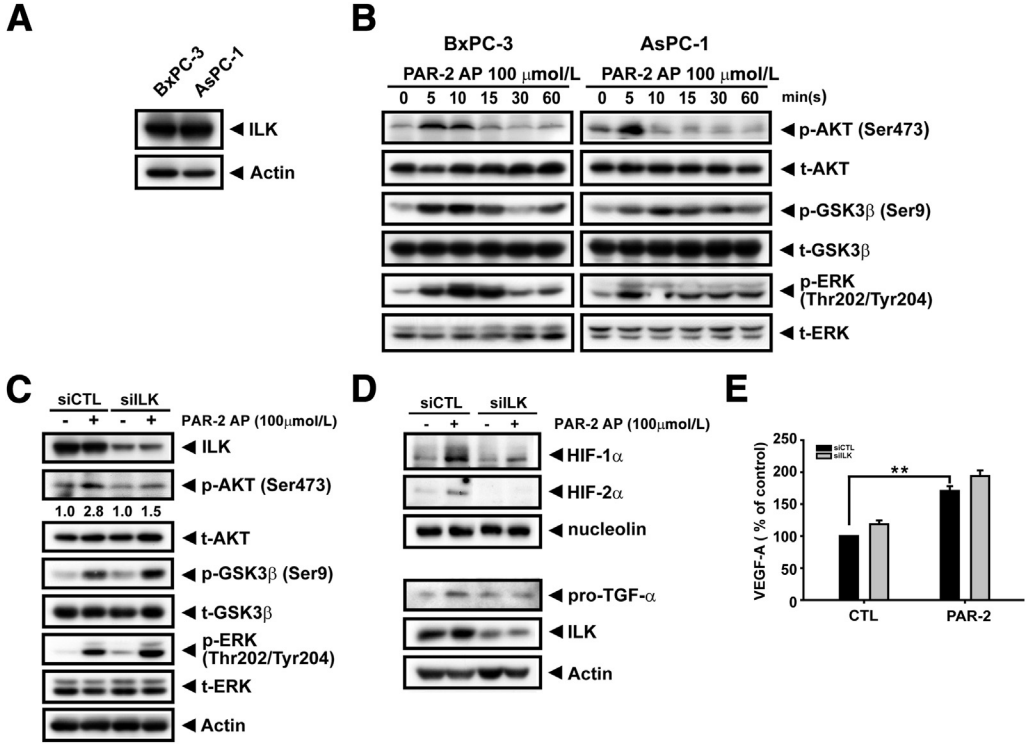


Figure 4 ILK signaling participated in PAR-2—mediated HIF-α and TGF-α regulation, but not VEGF-A regulation. Whole cell lysate or nuclear extracts were subjected to immunoblot analysis, as follows. **A**: BxPC-3 and AsPC-1 cells cultured in the culture medium. **B**: BxPC-3 and AsPC-1 cells treated with 100 μmol/L PAR-2 AP for different time intervals. **C**: BxPC-3 cells treated with or without 100 μmol/L PAR-2 AP for 10 minutes. **D**: Nuclear extracts from BxPC-3 cells treated with or without 100 μmol/L PAR-2 AP for 4 hours (**top panel**) or whole cell lysate from BxPC-3 cells treated with or without 100 μmol/L PAR-2 AP for 24 hours (**bottom panel**). **E**: VEGF-A levels in the culture medium from PAR-2 AP-treated BxPC-3 cells collected after 24 hours, as measured using an ELISA kit. The p and t represent phosphorylated and total, respectively. The mean values of *p*-AKT fold change, normalized by t-AKT, were shown below the panel. $^{**}P < 0.01$. CTL, control.

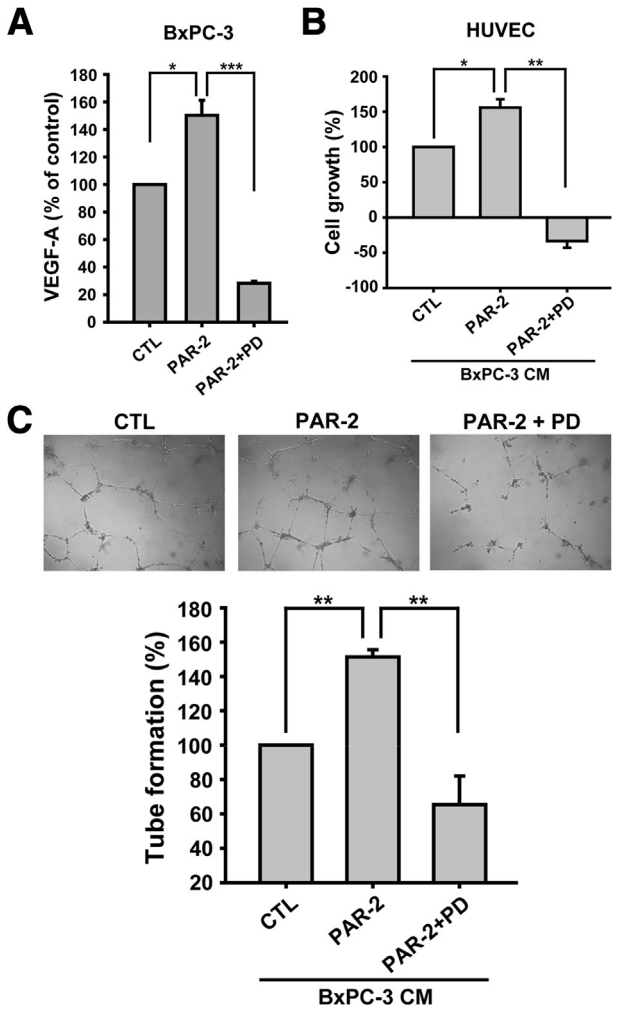


Figure 5 VEGF-A—regulated HUVEC growth and tube formation induced by PAR-2 AP is disrupted by the MEK inhibitor, PD98059. **A:** BxPC-3 cells were pretreated with 20 μmol/L PD98059 (PD) for 30 minutes, followed by 100 μmol/L PAR-2 AP for 24 hours. Culture media (CM) were collected from BxPC-3 cells, and VEGF-A levels were determined using an ELISA kit. **B:** HUVECs were treated with CM for 48 hours. Cells were stained with crystal violet, and cell growth was determined by measuring absorbance at 550 nm. **C:** *In vitro* Matrigel tube formation assay. **Top panel:** HUVECs were suspended in CM and seeded onto Matrigel-coated wells for 24 hours. Images of tube formation are shown. **Bottom panel:** The quantification of tube formation was performed using ImageJ software. Data represent means ± SEM from three independent experiments. CTL, control. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

In our previous study, signaling pathways induced by GPCRs, such as the AKT/mammalian target of rapamycin pathway, promoted the translation of HIF-α protein under normoxic conditions.[16] Herein, we demonstrated that activated PAR-2 by PAR-2 AP or FVIIa increased the protein expression of both HIF-1α and HIF-2α in human pancreatic cancer cells (Figure 2, A—C). We also found that both the BxPC-3 and AsPC-1 human pancreatic cancer cell lines expressed TF and PAR-2 mRNA (Figure 1A). Therefore, PAR-2 signaling certainly increased HIF-α protein expression in pancreatic cancer cells. Consistent with the results of our microarray analysis, both TGF-α and VEGF-A mRNA and protein levels increased after treatment with PAR-2 AP (Figure 2, D—F). *TGF-α* and *VEGF-A* are common target genes of HIF-1α and HIF-2α. The silencing of HIF-1α and HIF-2α, or treatment with the HIF-α inhibitor, YC-1, depleted TGF-α protein levels (Figure 3, B and D). However, the silencing of neither HIF-1α nor HIF-2α attenuated PAR-2 AP-induced VEGF-A production (Figure 3C). We, therefore, suggest that the activation of PAR-2 signaling stimulates TGF-α protein expression through an HIF-1α— and HIF-2α—dependent pathway.

TGF-α, an EGFR ligand, selectively binds to EGFR and activates EGFR-mediated signaling pathways, which regulate cell proliferation, differentiation, cell motility, and survival.[23] The up-regulation of TGF-α after HIF activation directly causes autocrine EGFR signaling.[24] In human pancreatic cancer, the overexpression of TGF-α cooperates with tumor suppressor, p53, to induce secondary genetic changes[25] and constitutively activates EGFR signaling to produce a gefitinib-effective phenotype.[26] Hence, our data suggest that PAR-2—mediated TGF-α expression regulates cross talk with EGFR signaling and promotes pancreatic cancer progression.

Much research is focused on the HIF switch. HIFs play a biological role in the hypoxic tumor microenvironment; acute hypoxia causes a rapid response and increases HIF-1α transactivation, thereby promoting angiogenesis to obtain oxygen. In contrast, chronic hypoxia mediates HIF-2α—dependent transactivation to remodel blood vessels and improves cell adaptation.[14] The results of this study have demonstrated that the depletion of HIF-1α by HIF-1α siRNA enhanced the nuclear expression of HIF-2α, with or without PAR-2 AP treatment (Figure 3A). We, therefore, speculate that HIF-2α is an important transcription factor in PAR-2—mediated signaling and that unidentified mediators regulate the interaction between HIF-1α and HIF-2α. HIF-2α is also known to interact with SP1 and HDAC4 to enhance FVII gene transcription,[27] indicating that the activation and switch of HIF-2α may alter cellular functions and increase the incidence of cancer-related thrombosis. Both HIF-1α and HIF-2α up-regulate genes involved in proliferation, angiogenesis, and metastasis, and are associated with tumor progression in several types of cancers. We also tested whether activated PAR-2 could increase the levels of adrenomedullin, GLUT1, and hexokinase-2 mRNA (Supplemental Figure S1), which act to regulate tumor progression in various ways.

ILK is a serine/threonine protein kinase that can directly interact with the cytoplasmic domain of integrins and possesses kinase activity regulated by cell-matrix interactions.[28] TF has the ability to cross talk with β1 integrin,[29] which binds with ILK. Indeed, treatment with PAR-2 AP induced the expression of *p*-AKT (Ser-473) and *p*-GSK-3β (Ser-9), which are downstream substrates of ILK, although a decrease in ILK levels as a result of siRNA treatment reduced PAR-2 AP-induced *p*-AKT (Ser-473) but not *p*-GSK-3β (Ser-9) expression (Figure 4C). In the results of this study, ERK is also not regulated by ILK. A study has indicated that ERK-primed inhibition of GSK-3β (*p*-Ser9) activity has been shown to be regulated by growth factors.[30]

We, therefore, suggest that *p*-GSK-3β (Ser-9) is phosphorylated through the ERK/p90RSk pathway, rather than through activated ILK. We further demonstrated that the inhibition of the ILK-AKT pathway by siILK resulted in the attenuation of HIF-α–induced TGF-α production, but not VEGF-A release (Figure 4, D and E). Compared with human prostate cancer cells,[10] our results indicate that the ILK-AKT pathway directly increases TGF-α production via an HIF-1α– and HIF-2α–dependent pathway in human pancreatic cancer cells. A previous report has shown that IL-1α promotes ILK kinase activity, leading to pancreatic cancer progression and poor patient survival.[31] We also found that the activation of PAR-2 induced the expression of *IL-1α* and other related genes, such as those encoding IL-1 receptor type 1 and toll-like receptor 4, and regulated the NF-κB pathway (Supplemental Table S1). This increase in IL-1α expression may, therefore, cause a positive feedback, resulting in enhanced ILK activity and downstream signaling in pancreatic cancer cells.

Tumor angiogenesis involves multiple processes, including interactions between tumor cells and the tumor microenvironment. Herein, we found that PAR-2 AP induced the release of VEGF-A, which promotes angiogenesis, in human pancreatic cancer cells and that this could be attenuated by the MEK inhibitor, PD98059 (Figure 5). This is similar to the results obtained for human breast cancer cells[5] and human glioblastoma cells.[6] According to our microarray analysis, the activation of PAR-2 signaling induced the expression of *MAPK1*, *ETS-1*, and *c-JUN*, which correlated with increased VEGF-A production. The proto-oncogene, Ets-1, which is regulated by *p*-ERK,[32] is known to control the expression of VEGF-A[33] and VEGF receptor 2, and it enhances tumor progression and promotes ECs to an angiogenic phenotype through their up-regulation. In this study, PAR-2 AP-treated tumor-conditioned medium significantly enhanced HUVEC proliferation and tube formation, which could be attenuated by using a conditioned medium containing the MEK inhibitor, PD98059 (Figure 5, B and C). MEK, as a direct signal mediator of *p*-ERK, therefore, induces angiogenesis through the activation of PAR-2 and subsequent VEGF-A release from human pancreatic cancer cells.

Collectively, our results have shown that PAR-2 signaling has effects on tumor cells and the tumor microenvironment and that these effects act to promote pancreatic tumor progression. First, the activation of PAR-2 enhances the expression of both HIF-1α and HIF-2α, which leads to increased TGF-α expression via ILK signaling, and it ultimately disposes pancreatic cancer cells to adaptation and progression. Second, MAPK plays a major role in controlling PAR-2–induced VEGF-A release and, consequently, angiogenesis. In conclusion, our experiments provide evidence that TF/FVII/PAR-2 signaling plays various roles in the development of pancreatic cancer, and it may provide an explanation for the correlation between pancreatic cancer and thrombosis.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.04.022*.

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